Phenotypes and mechanisms in myoclonus-dystonia
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Chapter One

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Aim and outline of this thesis
Myoclonus-dystonia: classification and phenotypic characteristics

Myoclonus-dystonia (M-D) is a neurological movement disorder classified as dystonia-plus syndrome, which describes a monogenic, likely primary form of dystonia with additional neurological manifestations such as myoclonus. Patients with M-D present with a combination of dystonic features and brief lightning-like myoclonic jerks that mostly affect the upper proximal extremities and the trunk. Myoclonic jerks generally represent the dominant and disabling feature and dystonia appears to be mild and mainly manifests as cervical dystonia and writer’s cramp. However, the phenotype is highly heterogeneous within or between families and disease severity or disease progression cannot be predicted. M-D is a rare disorder with unknown prevalence. It is inherited in an autosomal dominant manner with a reduced penetrance. Symptoms manifest in the first or second decade of life. In many cases the involuntary movements are reduced by alcohol consumption. Several studies reported psychiatric comorbidity in M-D families such as anxiety disorders, depression, addiction or obsessive-compulsive disorder. A recent survey of published literature revealed a significant association of psychiatric abnormalities in M-D, suggesting that they form part of the phenotype. A causative link remains to be demonstrated in a larger M-D cohort.

Genetic basis of myoclonus-dystonia

The epsilon-sarcoglycan gene and genetic heterogeneity

A major gene locus for M-D was mapped to human chromosome 7q21-31 and mutations in the gene epsilon-sarcoglycan (SGCE, DYT11) were found to be causative for the disease. Various types of SGCE mutations have been reported in several M-D families and sporadic cases such as missense, nonsense and splice site mutations, as well as small deletions. Large heterozygous deletions of the whole SGCE gene or of single exons have been reported recently. However, in many M-D patients no SGCE mutation was identified. In literature, only 23% of M-D cases were reported with a SGCE mutation. The lack of comprehensive clinical criteria for M-D and the phenotypic variability of different dystonia syndromes were thought to be the reason for the low percentage of SGCE mutation carriers. The introduction of classification criteria for M-D phenotypes (grouping into definite, probable and possible M-D) showed that SGCE mutation carriers are mainly presenting with a definite and probable form of M-D (definite M-D: early-onset myoclonus and dystonia or isolated myoclonus predominantly in the upper body half and a positive family history for myoclonus and/or dystonia; probable M-D: same criteria, but a negative family history). Still, there are a considerable number of clinically typical, familial SGCE mutation-negative
In one family that was clinically indistinguishable from families with \textit{SGCE} mutations, linkage was found on chromosome 18p11, suggesting that there is genetic heterogeneity (DYT15). However, further refinement of this locus to a 3.18 MB region and subsequent sequencing and assessment for larger deletions or duplications of all known and predicted genes of this region has not yet resulted in the identification of disease causing mutations. Interestingly, patients with an 18p deletion syndrome have been reported with dystonic features as well as involuntary myoclonic jerks.

Two other genes have been associated with M-D. In one \textit{SGCE} mutation-positive M-D family a missense mutation in the D2 dopamine receptor gene (\textit{DRD2}) co-segregated with the disease and was not found in 500 controls. However, other studies did not find an association of this \textit{DRD2} missense change in other M-D families or sporadic cases and functional assays failed to show whether this amino acid change is a rare and benign variant or a functional mutation.

Another \textit{SGCE} mutation-positive M-D family was reported with an additional 18-bp in-frame deletion (F323_Y328del) in the \textit{DYT1} gene (\textit{TOR1A}), which is associated with early-onset generalised dystonia. It remains unclear whether this mutation has a pathogenic effect. Interestingly, one family member carried the 18-bp \textit{DYT1} deletion, but not the \textit{SGCE} mutation and presented with only dystonic symptoms without myoclonic features. This suggests that \textit{SGCE} is the major M-D associated gene in this family.

\textbf{Reduced penetrance due to maternal imprinting}

Myoclonus-dystonia inheritance follows an autosomal dominant pattern with a reduced penetrance. \textit{SGCE} is a maternally imprinted gene, which explains that symptoms only manifest upon paternal transmission. Maternal imprinting was shown for the murine as well as human gene. Both human studies demonstrated that the \textit{SGCE} promoter region is differentially methylated. The maternal allele showed methylated cytosines at all CpG dinucleotides in patient leukocytes and control brain tissue, which is associated with gene silencing. In one patient biallelic \textit{SGCE} expression and an incomplete methylation pattern have been identified. This partial loss of methylation may account for the observed biallelic expression and may be more frequent. In literature, there are few reports of M-D patients inheriting the mutation from their mother. In addition, an fMRI study revealed mild abnormalities in cerebral activation patterns of clinically asymptomatic, maternally inherited \textit{SGCE} mutation carriers (4 cases). In mice, \textit{Sgce} is only expressed from the paternal allele, except for a weak maternal expression in brain. However, this leaky expression was not verified in a second mouse study and could also not be shown in human control brain. A leaky expression or partial loss of imprinting may be a rare epigenetic variation explaining disease manifestation upon maternal transmission in a few cases.
Epsilon-sarcoglycan gene structure
The human SGCE gene was initially described having twelve exons, including three alternatively spliced exons (exon 2, 8, and 10, also called 9b, Figure 1). In addition, two alternatively spliced exons were identified in mouse studies: exon 11b and 11c, which is an extended version of exon 11b. Both are regulated in a tissue-specific manner and were only identified in the brain. However, the isoform containing exon 11c has not been verified at the protein level yet. The presence of exon 11b or 11c results in a frameshift, which leads to a different C-terminus, the intracellular part of the SGCE protein. Data about the function of different SGCE isoforms are yet not available.

Figure 1. Overview of annotated SGCE isoforms. Constitutive exons are depicted in black, alternatively spliced exons in grey. NM_003919 represents the ubiquitously (ub) expressed variant. NM_001099401 includes alternatively spliced exon 10 and has been reported as rare variant. NM_001099400 is a brain-specific (bs) variant comprising the alternatively spliced exon 11b. Relative positions of the exons and sizes of exons are correct, but exon sizes are not represented in the same scale as the introns.

Epsilon-sarcoglycan protein and the sarcoglycan complex
The human ubiquitously expressed SGCE is a 437-amino acid and type 1 transmembrane protein (Figure 2). It has a 46-amino acid hydrophobic N-terminal signal sequence that targets SGCE to the plasma membrane. The extracellular domain, the N-terminus, contains an asparagine residue for glycosylation (Asn200, exon 5) and four conserved cysteine residues (Cys235, Cys248, Cys258, Cys271, exon 6). The N-terminus is followed by a 23-amino acid hydrophobic transmembrane domain and the cytoplasmic C-terminus. The intracellular domain contains three consensus sites for phosphorylation; one for casein kinase II, one for protein kinase C, and one for both kinases. The brain-specific isoforms containing exon 11b or 11c have altered C-terminal amino acid sequences, creating a PDZ-binding motif, which is a protein-protein interaction domain. A cadherin-like domain has been identified in
the extracellular domain of SGCE. Cadherins play a role in cell adhesion and are dependent on calcium ions to function. No other domains have been identified.

SGCE is a member of the sarcoglycan family, a family of plasma membrane glycoproteins. Thus far, six different sarcoglycans have been identified (SGCA, -B, -D, -E, -G, -Z; Figure 2). Sarcoglycans form a heterotetrameric complex that associates with the dystrophin-associated glycoprotein complex (DGC) at plasma membranes. The DGC itself consists of different complexes: 1) the cytoplasmic subcomplex composed of dystrophin, the dystrobrevins, and the syntrophins; 2) the dystroglycan complex; and 3) the sarcoglycan complex. The DGC links the cytoskeleton (dystrophin binds to actin) to the extracellular matrix (alpha-dystroglycan binds to laminin alpha2), and is therefore thought to be important for the mechanical integrity of tissue and it provides a scaffold for signalling molecules (Figure 3). The precise function or role of the sarcoglycan complex in the DGC is not known, but its proper assembly and trafficking is crucial for DGC function: mutations in a sarcoglycan lead to lack or absence of other sarcoglycans, which has an effect on DGC stability and function. The composition of the DGC and its subcomplexes varies among tissues: SGCA, -B, -D, and -G are the major components of the sarcoglycan complex in skeletal muscle; in Schwann cells it is SGCB, -D, -E, and -Z. Different studies suggest that SGCB and SGCD are the essential components of the sarcoglycan complex with SGCA/E and SGCG/Z as the variable subunits. This is in line with the observation that SGCA and SGCE are highly homologous with a highly conserved exon-intron structure and 43% sequence identity (62% similarity) at protein level. The DGC complex in brain has not been characterised yet, but considering the expression pattern of individual sarcoglycans the composition as seen in Schwann cells has been predicted.

SGCE appears to be an atypical sarcoglycan, despite its sequence homology to SGCA. Mutations in SGCA, -B, -D and G lead to different forms of limb-girdle muscular dystrophy (LGMD), also called sarcoglycanopathies that are characterised by progressive muscle weakness. Mutations in SGCE have not been identified yet. Why mutations in SGCE lead to the exclusively CNS phenotype remains unclear and is one of the major topics addressed in this thesis. No muscular abnormalities were identified in M-D patients after morphological and immunohistological investigations, as well as clinical assessment of muscle strength and mass. Overexpression of Sgce in a Sgca knockout mouse model of LGMD resulted in a stable DGC complex in skeletal muscle with Sgce as a component of the sarcoglycan complex and ameliorated the phenotype. A recent study confirmed this finding, suggesting that Sgce can compensate Sgca deficiency in mice. It is unlikely that overexpression of SGCA can compensate for lack of SGCE in M-D as SGCA expression is restricted to muscle.
Figure 2. Schematic illustration of the six sarcoglycans (SGCA, -B, -D, -E, -G, -Z). For SGCE two isoforms are shown: brain-specific (NP_003910) and ubiquitous SGCE (NP_001092870); the altered intracellular C-terminal amino acid sequence of brain-specific SGCE is highlighted in light grey. Homologies at the amino acid level are indicated: SGCA and SGCE share 62% similarity, SGCG and SGCD 69%, SGCZ and SGCD 57% and SGCZ and SGCG 56%. SGCB shows weak homology to SGCG, SGCD, and SGCZ. SGCA and SGCE are type I transmembrane proteins and SGCB, SGCD, SGCG, and SGCZ are type II transmembrane proteins. The transmembrane domain is shown in dark grey. The highly conserved cysteine residues are represented in black filled circles. This figure was adapted from Hack and colleagues.59
null mice showed that the absence of Sgca resulted in loss of expression of the sarcoglycan complex in skeletal and cardiac muscle and no change in Sgce expression.\textsuperscript{57} In heterologous cells Sgce does not require other sarcoglycans to reach the plasma membrane.\textsuperscript{58} These findings suggest that Sgce does not need other sarcoglycans for its expression and that it is not (only) part of the sarcoglycan complex in muscle, but may form part of a distinct complex. Conversely, in Sgcb and Sgcd knockout mice all sarcoglycans, including Sgce, were absent or greatly reduced in muscle.\textsuperscript{59,60} However, as mutations in other members of the DGC lead to disruption of the whole complex and a muscular dystrophy syndrome without M-D symptoms, and as M-D patients do not show any muscular abnormalities,\textsuperscript{54} it is likely that SGCE acts independently of other sarcoglycans and does not cause DGC dysfunction.\textsuperscript{44} It seems evident that there is a gain of SGCE function in brain, e.g. through reported brain-specific isoforms, and redundancy for SGCE in non-brain tissues, explaining the observed CNS phenotype and the lack of muscle involvement in M-D patients.

**Epsilon-sarcoglycan expression pattern**

Although SGCE is highly homologous to SGCA, its expression pattern is notably different. SGCA expression is largely restricted to striated (cardiac and skeletal) muscle and appears at a relatively late stage of myogenesis.\textsuperscript{61-63} SGCE is more broadly expressed; it was detected in lung, liver, kidney, heart, muscle, testis, pancreas, thymus, intestine, spleen, brain, and peripheral nerve.\textsuperscript{41,42} In general, SGCE expression was higher in blood vessels and nerves compared to muscle fibers,\textsuperscript{42} and it was largely restricted to the cytoplasm and proximal processes.\textsuperscript{64} Throughout the brain SGCE was predominantly detected in the cerebellum, putamen, cerebral cortex, hippocampus, pons, and olfactory bulb.\textsuperscript{12,65} In mouse brain, high expression was also observed in monoaminergic neurons of the brain stem and hypothalamus.\textsuperscript{64} SGCE was expressed in embryos, suggesting a possible role during embryonic development.\textsuperscript{42,65} One study showed neuronal and non-neuronal expression (capillary endothelial cells and astrocytes) in the mouse brain.\textsuperscript{41} The same group showed that the ubiquitous and the brain-specific isoform (with the inclusion of exon 11b) have different cellular localisation. The ubiquitous form was enriched in postsynaptic fractions, whereas the brain-specific isoform was enriched in the presynaptic membrane fraction, suggesting (1) different roles for different SGCE isoforms and (2) a role for SGCE in synapse function.

Of all sarcoglycans, SGCZ is the only sarcoglycan that shows the ubiquitous expression pattern of SGCE including expression in the brain.\textsuperscript{66} This observation makes it an interesting candidate gene for SGCE mutation-negative M-D patients. However, we did not find mutations in the coding region of SGCZ in this patient cohort yet (unpublished data).
Figure 3. The integral and peripheral components of the dystrophin-associated glycoprotein complex (DGC) and other membrane-associated proteins and binding partners in skeletal muscle. Abbreviations: ABD, actin-binding domain; EF1/EFII, EF hand domains; H1/H2, helical domains; nNOS, neuronal nitric oxide synthase; PDZ, PSD-95 (postsynaptic density protein 95); PH, pleckstrin homology domain; SAST, syntrophin-associated serine/threonine kinase; SBD, syntrophin-binding domain; SSPN, sarcospan; SU, syntrophin-unique region. This figure was reprinted with permission from Waite and colleagues. 60

Pathophysiology of myoclonus-dystonia

Functional aspects

Little is known about pathophysiological mechanisms in M-D. Myoclonic symptoms in M-D are thought to be of subcortical origin due to lack of stimulus sensitivity and the absence of giant somatosensory evoked potentials.46 Dystonic symptoms in M-D point towards involvement of the basal ganglia. One study identified an abnormal common low frequency drive to the dystonic muscles in M-D patients by coherence analysis of EEG-EMG and EMG-EMG studies, and this observation is thought to be a result of basal ganglia dysfunction.68 Neurophysiological examinations, including TMS studies failed to show abnormal cortical involvement in M-D. With TMS, intracortical inhibition in M-D patients appeared to be normal.69,70 Severity of dystonic symptoms in M-D have been associated with an increased grey matter volume in the putamen in MRI studies of clinically affected SGCE mutation carriers.71 Dystonia in general
is thought to originate from dysfunction of the basal ganglia and/or cerebellar motor loop (Figure 4). A proposed model suggests that hyperactivity of the direct putamenv pallidal pathway leads to a reduced inhibitory output of the internal segment of the globus pallidus (GPi), which in turn leads to an increased thalamic input to the (pre) motor cortex, resulting in excessive stimulation of the motor cortex and abnormal movements. However, more and more evidence points towards involvement of the cerebellum in the pathophysiology of dystonia.

**Figure 4.** Schematic and simplified diagram of the basal ganglia-thalamo-cortical and the cerebellothalamo-cortical circuits. The basal ganglia circuit receives most of its input in the caudate and putamen, also called striatum (dopaminergic input from the substantia nigra (SN) and glutamatergic input from the cerebral cortex). The internal globus pallidus (GPi) and SN form the output stage of the putamen by means of two pathways: 1) the direct pathway sends inhibitory striatal projections directly to GPi and SN; 2) the indirect pathway sends inhibitory projections to GPi and SN by means of the external globus pallidus (GPe) and subthalamic nucleus (STN). The GPi sends inhibitory fibres to the thalamus, which in turn projects to the sensorimotor cortex. Primary motor cortex neurons project through the brain stem to the spinal motor neurons and respective muscles. The cerebellum receives cortical input via the pons and sends information back to the cortex via the thalamus, providing feedback to the motor cortex. Recently, a connection from the cerebellum (dentate nucleus) to the striatum and subsequently to the GPe has been reported as well as projections from the STN to the cerebellum providing a direct route for cerebellar activity to influence and be influenced by the basal ganglia activity (not shown). Abbreviations: CbC, cerebellar cortex; CM, thalamic centromedian; DCN, deep cerebellar nuclei; PN, pontine nuclei; PPN, pedunculopontine nucleus; SNC, substantia nigra compacta; SNr, substantia nigra reticulata; VA/VL, ventral anterior/lateral nucleus of the thalamus; Vim, ventral intermediate nucleus of the thalamus. This figure was adapted from Kringelbach and colleagues.
Functional imaging studies have been performed in a limited number of M-D patients. An \(^{18}\)FDG-PET study in one \textit{SGCE} mutation carrier revealed hypermetabolism in the ventrolateral thalamus as well as bilateral spinocerebellar regions.\(^{76}\) A SPECT study in a small group of M-D patients revealed reduced dopamine D2 receptor availability in the striatum, which could hint towards increased dopamine levels and subsequent downregulation of the receptor.\(^{77}\) fMRI studies of genetically confirmed M-D patients showed abnormal activation of thalamus and cerebellum (dentate nucleus) in a single case,\(^{78}\) and significant hyperresponsiveness in inferior parietal cortical areas, premotor and primary somatosensory cortex and in the cerebellum in thirteen cases.\(^{79}\)

**Molecular aspects**

The predicted \textit{SGCE}-associated disease mechanism is loss of function owing to one imprinted and one mutated allele. Nonsense mutations and mutations leading to a premature stop codon are predicted to be degraded by nonsense-mediated decay.\(^{80}\) Also \textit{SGCE} missense mutants are subject to degradation as suggested by \textit{in vitro} studies, showing that these mutants were not transported to the plasma membrane in cortical neurons, but were retained intracellularly and degraded by the proteasome.\(^{58}\) The predicted loss of function of different types of \textit{SGCE} mutations provides an explanation for the lack of phenotype-genotype correlations in M-D.\(^{19}\)

There is no data on \textit{SGCE} function. One M-D animal model has been generated thus far. Yokoi and colleagues described a paternally inherited heterozygous \textit{Sgce} knockout mouse model.\(^{81}\) These mice lack exon 4 resulting in a premature stop codon in exon 5. Its mRNA is predicted to be unstable and targeted by nonsense-mediated decay. These paternally inherited heterozygotes lack Sgce protein, as \textit{Sgce} is a maternally imprinted gene. The phenotype of these mice highly resembled M-D symptoms: they presented with myoclonus, deficits in motor coordination, balance, and learning, and psychiatric alterations that were consistent with anxiety and depression. \textit{Sgce} knockout mice do not manifest dystonic symptoms. Further studies revealed an altered monoamine metabolism with a significant increase of dopamine levels in the striatum of these animals. This is in line with the observed reduced striatal D2 receptor binding in SPECT studies in M-D patients that can be explained by increased dopamine levels.\(^{77}\) Interestingly, overexpression of torsinA affects Sgce clearance \textit{in vitro};\(^{58}\) a 3-bp deletion in \textit{TOR1A} is responsible for a different form of dystonia (DYT1 dystonia). However, it is not known whether this interaction occurs \textit{in vivo} and whether this link is related to a common disease mechanism.
Therapy

There is no cure for M-D; only symptomatic drug treatment can be performed with moderate or no effect. Pharmacological treatment normally involves benzodiazepines, antiepileptic drugs, anticholinergics, dopaminergic agents or GABA agonists. Dystonic symptoms, especially focal dystonias, can successfully be reduced by botulinum toxin injections. The toxin ameliorates symptoms by blocking the release of acetylcholine into the neuromuscular junction.

Deep brain stimulation (DBS) is another treatment option for patients with a severe phenotype if oral medication does not alleviate symptoms or lead to intolerable side effects. Single case reports and small studies reported DBS in bilateral GPi as well as ventral intermediate thalamic nucleus in patients with confirmed SGCE mutations. Myoclonic and dystonic symptoms showed a marked improvement in all studies (>50%). Little is known about long-term side effects of DBS, but psychiatric complications have been reported occasionally. A small number of dystonia patients committed suicide after GPi stimulation; it remains unclear whether DBS triggers these symptoms. This side effect should be taken into account especially in M-D patients, as patients with a history of depression may be at higher risk.

Aim and outline of this thesis

The pathophysiology of M-D is largely unknown. The major aims of this thesis are to get a better understanding of the role of SGCE in M-D by (1) defining its phenotypic spectrum, (2) the identification of brain regions affected in M-D, and (3) elucidating SGCE protein function.

As dystonic symptoms in M-D generally manifest as focal dystonia and frequently as writer’s cramp, we investigated the prevalence of SGCE mutations among patients with writer’s cramp as primary symptom in chapter 2. In chapter 3 the importance of strict classification criteria in M-D and the role of SGCE gene dosage analysis are assessed and discussed. Chapter 4 addresses the major research question, why loss of SGCE, a protein that is expressed in several tissues, leads to exclusively neurological symptoms and aims at the characterisation of brain regions affected in M-D. We provide evidence that the major brain-specific SGCE isoform plays a role in the cerebellum, and hypothesised a role for this isoform in motor coordination. To get insights into SGCE function, we then studied protein-protein interactions of this isoform in chapter 5. In chapter 6 all findings are summarised and discussed, and implications and future research questions are presented.
Part 2 of this thesis describes research related to the SGCE isoforms characterisation study presented in chapter 4. An in-depth analysis of ultra-deep sequencing data of this study revealed many novel low frequency variants that cannot be explained by alternative splicing. These variants are presented and characterised in chapter 7 and implications are further discussed in chapter 8.

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